TRANSLATING THE ROLE OF EXTRA-HEPATIC MANIFESTATIONS IN PATHOGENESIS OF HEPATITIS B VIRUS

SHORT SYNOPSIS

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by

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ABSTRACT

The Hepatitis B viral infection is a pathological pandemic. One of the most evident causative factors of liver disease, the hepatitis B virus is a model virus from the *Hepadnaviridae* family. The global mortality rate owing to the infection related complications has reached to near about 1 million. The progressive rise in the cases of viral Hepatitis is immensely burdening the healthcare systems. Known for its hepatotropism, liver is the prime target of infection for this virus. Much of the research till date has been dedicated to the elucidation of the virulent pathways of the virus in association to the liver and associated diseases by utilizing the viral host protein interaction networks. This in turn has facilitated avenues to generate effective vaccines and therapeutics for successful prevention of infection and for managing the symptoms. However, the complete eradication of the virus from the host is still complicated due to the lack of complete picture of the precise ways of the viral pathogenesis. Aspects of virulence besides the hepatocytes have been ambiguous yet observed in many studies indicating a significant contribution to the viral pathogenesis. Protein interaction studies between the leukocytes and two of HBV proteins have confirmed the missing interaction but the studies have not been validated. The lack of unbiased data and the knowledge of the exact mechanism and function of the leukocytes with respect to the virus demands a systematic study of Protein Protein interaction (PPI) generation and analysis. Therefore, a study has been designed to generate a viral host interactome between the viral proteins and the host leukocytes. This interactome would highlight the key proteins of the host leukocytes traced by the virus to manifest the infection successfully, thereby opening new avenues of research in the field of hepatitis B viral infection.

Key words: Hepatitis B virus, Protein-Protein Interactions, Leukocytes, yeast two hybrid system.

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INTRODUCTION

Hepatitis B has chronically infected a population of near about four hundred million across the globe (Lin and Zhang; 2017). India, being intermediately endemic, has a disease burden of nearly around fifty million. In India, Hepatitis B related diseases is becoming at par with the major diseases such as tuberculosis and malaria (Satsangi and Chawla, 2016).
According to World Health Organization, Hepatitis B virus is accredited with being amongst the prime causal factors of severe liver related illness and is considered the tenth leading cause of death (WHO Report, 2017). The infection caused by the virus has the potential of initiating life threatening conditions of liver such as hepatocellular necrosis and liver cancer. The infection may manifest either as acute with febrile symptoms or as chronic infection thereby persisting without any symptoms for long periods of incubation in the host. The complication of chronic form of infection results in liver cirrhosis and hepatocellular carcinoma (HCC). Somewhere about 20 to 30% of those chronically infected is likely to develop fatal complications due to progressive liver related illnesses. An estimated annual mortality rate due to chronic HBV infection is about six lakh fifty thousand (Liang, 2009).

Many years of research dedicated exclusively to HBV have resulted in substantial increase in knowledge of various aspects of HBV pathogenesis. Owing to this, effective vaccines have been generated to avert infection in the naive population. The availability of vaccines against Hepatitis B virus has been there since 1982 (Liao and Liang, 2015). The vaccines are successful in demonstrating the efficacy of 95% in prevention of infection and hence the onset of chronic liver disease which would lead to possible hepatocelular carcinoma. However, in case of mute chronic infection, the silent presence of viral and sub viral particles render the administered vaccine ineffective. On the other hand, the therapeutics for treating HBV such as pegylated interferon alpha, tenofovir, entecavir; so developed are generally useful in terms of immediate relief from the symptoms. The effect of therapeutics is short term and requires a prolonged use to avert the relapse of infection. The discomfort due to side effects provided by the therapeutics and medical expenditure borne by the infected makes its long term affordability a grave challenge. This leads to untimely discontinuation of drug thereby increases the possibility of relapse (Gish et al, 2012).

1.1) OVERVIEW OF HEPATITS B PATHOGENESIS

Hepatitis B virus from *Hepadnaviridae* family has a relaxed circular, partially double-stranded DNA with genome size of about 3.8 Kb, consisting of a core nucleocapsid with core antigen (HBcAg), enwrapped by an outer envelope embedded with the surface antigen (HBsAg) (Ganem, 2001). The viral genome encodes four major open reading frames (Grimm, Thimme and Blum, 2011):
1. The precore/core gene, coding for the nucleocapsid protein and the precore protein (hepatitis B e antigen (HBeAg)).
2. The polymerase gene, coding for the reverse transcriptase/ HBV polymerase.
3. The PreS1/L, PreS2/M, and Surface/S genes, coding for the three envelope proteins.

4. The X gene, coding for the regulatory X protein.

The virion reaches the target site by circulating in the body fluids and penetrates the hepatocytes of the liver by interacting with Sodium taurocholate cotransporting polypeptide also known as bile acid transporter (Huang et al, 2012). The virion uses the cytoskeleton for mobility within the host cell. Once in the cytoplasm, the virion makes its way towards the nucleus and injects the relaxed circular DNA into the nucleus. The partially double stranded relaxed circular DNA is then repaired by the viral polymerase to form a covalently closed circular DNA (cccDNA), thus imparting uniquely high stability to this episomic form of viral DNA. It's usually the persistence of this episomic form of DNA that makes the complete eradication of viral load from the host system a daunting task. Moreover, the replication of HBV genome is highly prone to errors especially in the precore region which adds to the additional burden in detection and eradication of virus (Lamontagne, Bagga and Bouchard, 2016).

CURRENT STATUS OF VACCINES AND THERAPEUTICS

At present, nearly 180 countries have understood the relevance of HBV vaccination and have taken the initiative to incorporate HBV vaccines to their pre-existing routine vaccination programs. One of the most important steps towards immunization was administration of HBV vaccines in the neonatal population usually in a time period of less than twenty four hours of birth. Such a vaccine program has been the first endeavour in public domain against HBV and this has been the first anti-cancer program to be initiated. The major goal of vaccine development is to prevent the further spread of virus and to protect the population prone to future infection due to unwarranted contact with the infected person or environment (Terrault, 2009). However, the development of vaccines has proved to be a great initiative for only that section of population which is unaffected by the virus in any form. The vaccines however are rendered useless for the population which is inflicted by the virus (McMahon. 2005). In the recent times, development of a mammalian cell derived recombinant vaccine has successfully been accomplished. These vaccines have been classified into three groups depending on the type of antigen being used in the formulation as the prime effector molecule. Out of the three formulations, two vaccines are monovalent where the effectors are derived from Pre S1 and Pre S2 respectively, while the third vaccine formulation is bivalent and it utilises the combination of one epitope each derived from S and Pre S region. These vaccines have shown to be highly efficacious in eliciting an immune response which had conferred sufficient protection against the virus during the clinical trials (Lok, 2004).

At present, the Food and Drug Administration, USA; has given clearance for seven of the drugs to treat chronic hepatitis B. These seven drugs are: standard alpha interferon, PEG-interferon and five nucleotide analogs (Keeffe, 2008). The former treatment is based on the enhancement of the host's immune defenses against the virus and the later treatment leads to the inhibition with varying potencies of the viral DNA polymerase to prevent it spreading the infection further. Nucleotide analogs currently available are adefovir, entecavir, lamivudine, telbivudine and tenofovir (Zoulim and Locarnini, 2009).
However, a complete eradication of virus from the host is never achieved, rendering the host at risk of recurrence of infection later in life. Therefore a lifelong treatment is recommended for prevention of recurrence of viral infection. There is however a downside to all the treatments mentioned above. The primary treatment using the pegylated interferon treatment is sustained only in about thirty percent in the hepatitis B 'e' antigen positive cases whereas around forty percent in Hepatitis B 'e' negative antigen cases and that to laden with heavy discomforting side effects (Perrillo, 2009; Janssen et al, 2005; Lau et al, 2005). Nonetheless, considering the nucleoside analog treatment, they potentially hinder the HBV from progressing by suppressing their replication, thereby subsiding the infection considerably amongst the majority of patients treated (Rehermann et al, 1996). Yet this treatment also works till the time the therapeutic is being administered. None of the treatments facilitate a complete eradication of the virus from the host system. Hence a long term dependency on therapeutics or rather a life-long dependency becomes a necessity than a choice (Block et al, 2013). Additionally the cost of therapy as well as the toxicity caused due to continuous administration of the drug makes therapy less viable and feasible (Kim et al, 2013).

LITERATURE REVIEW

The Hepatitis B virus is known for causing chronic liver related diseases with fatal eventualities. Various viral components such as genetic material (DNA and RNA) as well as proteins and their subunits have been found in diverse range of extra-hepatic host sites which may include cerebro-spinal fluid, lymph nodes, brain, spleen, leukocytes and Peripheral Blood Mononuclear Cells (PBMCs). PBMCs are composed of varied components of blood such as erythrocytes, leukocytes and thrombocytes out of which leukocytes are the cells which are more prone to viral invasion as these contain a nucleus. PBMCs have been the choice of study owing to their ease of accessibility and feasible in-vitro sustainability (Coffin et al, 2015; Mei et al, 2000; Leung et al, 1994; Mason et al, 1993; Yoffe et al, 1990). Additionally, two medical incidences have reinforced a possibility of the immune cells acting as a source of causing re-infection in spite a successful liver transplant (Datta et al, 2009; Murakami et al, 2004).

The presence of the virus within the host cell indicates permissive interactions between viral and host systems. This facilitates the virus to manifest itself successfully thereby evading the host defense systems. Decoding the molecular basis of such interaction is done through the analysis of the viral host protein protein interaction networks. The Protein Protein Interaction (PPI) data generated can be utilized to either acquire a basic knowledge about the mandatory pathways utilized by the virion or to find out the effective target sites to be considered from the point of view of therapeutics. An unbiased and comprehensive PPI data can be scientifically exploited in many ways to explore the unanswered, for instance, a basic research was done to understand the proper mechanism of folding of the capsid proteins using weak protein interactions (Pablo et al, 2002). Another PPI study by demonstrated the contribution of HBV infection in subsequent development of hepatocellular carcinoma (Zhong-Jun Wu et al, 2010). A study to understand the imperative biomarkers in the Hepatitis B infection was carried out in 2015 using PPI studies by Thomas *et.al;* who tried to elucidate the HBV and HCV related biomarkers generated during the infection (Thomas et al, 2015). Similarly a study published in 2017 by Livingston *et.al;* identified and characterized the interplay between the viral protein X and host protein smc5/6 using protein interaction studies from the view of generating information for the future development of antiviral and therapeutics (Livingston et al, 2017).
Extra-hepatic prevalence of the virus has been depicted by the protein interaction studies of the virus with the host leukocytes. Positive protein interaction data between the host leukocytes and the viral core protein as well as viral preS1 protein have been demonstrated successfully (Lin et al, 2006; Zhang et al, 2006). However due to lack of validation studies the role of leukocytes in the pathogenesis yet remains inconclusive.
The apparent characterization of the HBV to be hepatotropic therefore becomes ambiguous and opens new avenues for research in order to boost the pre-existing measure taken for the eradication of this virus. Further deliberations are needed to establish a new line of research where preventives, diagnostics and therapeutics so developed must target the immune cells especially the leukocytes. Therefore, our study has been designed to identify, screen and validate the viral proteins interactions with the host leukocytes. The study has been designed to generate maximum interaction data between host leukocytes and virus so as to identify the key target sites essential for establishing the virulent pathways. These key interaction sites

may highlight the hub protein interactions necessary for the virus, which in turn would set the course of research for further characterization of the interaction.

DESCRIPTION OF BROAD AREA

Viral Hepatitis is increasingly becoming a public health concern in India, but due to lack of proper awareness and with much less priority for vaccination being given to prevent Hepatitis B; management of infection and timely corrective action becomes a grave challenge. In India itself near about forty million are chronically infected. The vaccinations have been available globally since a very long time but vaccination strategies are not at par with the current need of the hour and lack of logistics reduces the chances of successful implementation of vaccination programs across the nation. Effective antivirals for suppression of progression of the infection have been approved by the FDA but due to lack of HBV antigenic seroconversion, the possibility of relapse remains upon lack of proper and timely adherence to the prescribed program of therapy. In order to keep the infection under check the antiviral therapy is required for lifelong purposes. However, the cost of the therapy and thwarting side effects makes the adherence to scheduled therapy non-complaint therefore leading to the progression of the condition into more lethal forms such as liver failure or Hepatocellular carcinoma. These events lead to a huge burden on economy as well as healthcare.

Apart from the general therapeutics which follows the conventional route of the virus to disrupt the working mechanism of the virus, there are other avenues to be looked into which have not been explored completely. For instance, HBV is also found to harbor in the host cells other than hepatocytes of the liver such as host leukocytes. It is hypothesized that these cells have a major role in facilitating the viral persistence and transmission thus masking the presence of virus from the host defenses. Moreover, the studies exploring the leukocyte aspect of the HBV pathogenesis has highlighted the presence of the virions in the leukocytes time and again, and cases have been documented of the relapse of HBV infection despite of liver transplant. Yet the studies on leukocytes interaction with the HBV have not been conclusive; however appears to hold great promise in the direction of therapeutics from the indicated point of view. This can be achieved by generating a host viral protein protein interaction data between the host leukocytes and the viral proteins which have the potential to highlight the key proteins and their corresponding pathways necessary for successful pathogenesis. Identification of key interactors shall pave the way for new line of vaccines and therapeutics.

OBJECTIVES OF RESEARCH

- To clone and characterize viral ORFs in cloning vectors.
- To carry out the interaction interplay between the virus and host leukocytes.
- To validate the interaction data thus generated and identify the key interaction targets.

METHODOLOGY

The starting material for the project i.e., hepatitis B viral DNA will be provided by SRL diagnostics, Gurugram. The DNA will be cloned and sequenced subsequently for ensuring the purity and authenticity of the base material. Y2H Hybrid assay with the human leukocytes cDNA library would show schematics of host receptors utilized for the viral pathogenesis. The detailed work flow will be followed as:

A. Viral-Host protein interaction Analysis

(i) Construction of BD and AD fusion genes

(A) Construction of BD fusion genes: Viral Genes encoding proteins S, C, P and X; the identified significant effector molecules employed in HBV infection pathways will be cloned in BD vector (pGBKT7) of yeast two hybrid system.

(B) Construction of AD fusion genes: An AD fusion cDNA library in pGADT7-AD vector of human leukocytes cells will be prepared using manufacturer's kit.

(ii) HBV-ORF and host protein interaction in yeast

AD fusion plasmid library of human leukocyte cDNA will be transformed in a large scale protocol into the yeast cells previously transformed with HBV proteins containing BD vectors. After transformation, the positive interactions will be studied by blue-white screening and by selection under high stringency conditions using SD/-His-Leu-Trp/X-Gal auxotrophic media. The clones capable of growing on SD/-His-Leu-Trp will be saved and further subjected to stringency test using varying concentration of 3-AT (3-amino-1,2,4-triazole). The AD plasmids will be isolated from the selected yeast clones showing interaction. Identification of the interacting leukocyte cDNA-AD clones will be revealed by sequencing using 3´AD and T7 sequencing primers. The cDNA sequences obtained will be compared to those in GenBank, EMBL or other Databases for the identity of genes. Truncated interacting cDNA clones will be avoided and full length cDNAs will be selected from the library.

(iii) Validation of HBV-ORF and host protein interactions

The interactions obtained from yeast based screening will be further validated. For each HBV-ORF we will examine at least five positive host interacting proteins by

(A) GST pull down assay and/or

(B) Co-immunoprecipitation (Co-IP) assay

(A) **GST pull down assay**: HBV-ORFs will be cloned in pGEX4T3 vector, or other suitable variant plasmid with similar features. The proteins will be expressed as glutathione S-transferase (GST) fusions in E. coli strain BL21. The supernatant containing protein will be recovered after sonication and centrifugation and allowed to bind glutathione-sepharose (GSH) beads under suitable conditions. The putative binding cDNAs from AD vector will be sub-cloned into the pXJ40-FLAG vector and in vitro transcription and translation (IVT) will be

carried out. The IVT mixture will be passed through GST affinity column containing the HBV protein. After washing steps, bound protein will be dislodged from the beads by heating the sample in buffer, electrophoresed on SDS-PAGE gel and visualized by anti-FLAG antibody.
(B) **Co-immunoprecipitation (Co-IP):** HBV ORFs will be cloned in suitable restriction sites of pGEX4T3-GST for expression as GST-tagged proteins. Desired binding protein (obtained from Y2H screen) from the host will be expressed as FLAG-tagged protein from pXJ40-FLAG vector. Vero cells will be transfected with each of the pXJ40 encoding HBV-GST and host protein-FLAG. After 24-36 hours transfected cells will be harvested in lysis buffer and lysate will be prepared. Lysate will be incubated with GSH beads, followed by washing. Samples will be retrieved by boiling in SDS-PAGE sample buffer followed by separation on SDS-PAGE gel and blotting. The blots will be probed with anti-FLAG monoclonal antibody.

EXPECTED OUTCOME OF THE RESEARCH

It is expected that generation of host leukocyte and viral protein interactome shall highlight the pathway and receptors of leukocytes utilized by the virus to successfully establish an infection. Knowledge of such key pathways and receptors can help us identify targets which can be utilized for the disruption of virulence and development of effective therapeutics even before the virus reaches its target site and exploited by virus for further transmission.

The success of the proposed research shall open avenues in the applied field of research for generation of a more potent and effective curative therapeutic development which is the need of the hour ensuring complete eradication of Hepatitis B infection.

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